Improved Product-Per-Glucose Yields in P450-Dependent Propane Biotransformations Using Engineered Escherichia coli

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Received 11 July 2010; revision received 10 September 2010; accepted 18 October 2010
Published online 28 October 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22984

ABSTRACT: P450-dependent biotransformations in Escherichia coli are attractive for the selective oxidation of organic molecules using mild and sustainable procedures. The overall efficiency of these processes, however, relies on how effectively the NAD(P)H cofactors derived from oxidation of the carbon source are utilized inside the cell to support the heterologous P450-catalyzed reaction. In this work, we investigate the use of metabolic and protein engineering to enhance the product-per-glucose yield (YPgc) in whole-cell reactions involving a proficient NADPH-dependent P450 propane monooxygenase prepared by directed evolution [P450PMOR2; Fasan et al. (2007); Angew Chem Int Ed 46:8414–8418]. Our studies revealed that the metabolism of E. coli (W3110) is able to support only a modest propa-nol:glucose molar ratio (YPgc/C24 ~ 0.5) under aerobic, non-growing conditions. By altering key processes involved in NAD(P)H metabolism of the host, considerable improvements of this ratio could be achieved. A metabolically engineered E. coli strain featuring partial inactivation of the endogenous respiratory chain (Dndh) combined with removal of two fermentation pathways (DadhE, Dldh) provided the highest YPgc (1.71) among the strains investigated, enabling a 230% more efficient utilization of the energy source (glucose) in the propane biotransformation compared to the native E. coli strain. Using an engineered P450PMOR2 variant which can utilize NADPH and NADH with equal efficiency, we also established that dual cofactor specificity of the P450 enzyme can provide an appreciable improvement in YPgc. Kinetic analyses suggest, however, that much more favorable parameters (Km, kcat) for the NADH-driven reaction are required to effectively compete with the host’s endogenous NADH-utilizing enzymes. Overall, the metabolic/protein engineering strategies described here can be of general value for improving the performance of NAD(P)H-dependent whole-cell biotransformations in E. coli.

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KEYWORDS: cytochrome P450 monooxygenase; alkane oxidation; BM-3; whole-cell biotransformations; protein engineering; Escherichia coli

Introduction

The selective oxidation of unactivated C–H bonds is a challenging chemical transformation of great synthetic and industrial value (Li et al., 2002; Schmid et al., 2001). Cytochrome P450 monooxygenases represent attractive biocatalysts to carry out this task due to their ability to catalyze the oxygenation of allylic, aromatic, and aliphatic C–H bonds under mild reaction conditions (Denisov et al., 2005; Sono et al., 1996). Natural P450s and engineered variants of these enzymes have proven useful for various applications, including the preparation of drug metabolites (Guengerich, 2002; Sawayama et al., 2009) and synthetic intermediates (Bell et al., 2006; Landwehr et al., 2006), the oxidation of natural products (Chang et al., 2007; Sowden et al., 2005) and alkanes (Fasan et al., 2007; Peters et al., 2003; Xu et al., 2005) and for chemo-enzymatic synthesis (Lewis et al., 2009; Rentmeister et al., 2009).
To support P450-catalyzed oxidations, a supply of reducing equivalents in the form of reduced nicotinamide cofactors NAD(P)H is generally required. While this can be achieved in vitro using auxiliary cofactor regeneration systems (Johannes et al., 2008; van der Donk and Zhao, 2003), the use of whole-cell systems offers important advantages (Duetz et al., 2001; Li et al., 2002) as NAD(P)H cofactors can be regenerated through the oxidation of inexpensive biomass-derived sugars within a metabolically active cell (Faber, 2004; Li et al., 2002; Schmid et al., 2001). A key aspect of these processes is the net balance between the energy input (glucose) and product output. This parameter, which can be expressed as the molar ratio between the product formed and the glucose consumed ($Y_{PPG}$), ultimately depends on the efficiency with which the reducing equivalents derived from glucose catabolism are channeled towards the heterologous NAD(P)H-dependent reaction in the presence of various cellular processes competing for the same cofactor(s). In previous studies, product-per-glucose yields ($Y_{PPG}$) have been analyzed in the context of biotransformations using heterologous dehydrogenases and reductases (Chin et al., 2009; Cirino et al., 2006; Walton and Stewart, 2004). Much scarcer information is available about $Y_{PPG}$ achievable in *Escherichia coli* for whole-cell reactions involving P450 enzymes and, especially, about strategies to enhance the efficiency of glucose utilization in these processes.

Mild oxidation catalysts for the selective oxidation of gaseous alkanes are highly sought-after because of their potential utility for the conversion of natural gases into liquid fuels (Olah et al., 2006). To this end, we have recently evolved a proficient P450 propane monooxygenase (P450PMOR2) for the conversion of propane to propanol (Fasan et al., 2006). P450PMOR2 was derived from P450BM3, a self-sufficient P450 from *Bacillus megaterium* with high activity on long-chain ($C_{12}-C_{20}$) fatty acids (Narhi and Fulco, 1987). As a result of 23 amino acid substitutions and significant alteration of the active site configuration (Fasan et al., 2008), P450PMOR2 was found to possess “native-like” proficiency in propane oxidation (370 turnovers/min, 98% coupling) supporting more than 45,000 turnovers (Fasan et al., 2007). P450PMOR2 also showed promise as biocatalyst for whole-cell biotransformation of propane to isopropanol and 1-propanol in 9:1 ratio, providing in vivo activities up to 120 U g$^{-1}$ cell dry weight (cdw) using resting *E. coli* (DH5α) cells (Fasan et al., 2007). Isopropanol could be useful for the production of isopropylene (Kibby and Hall, 1972), as monomer for polypropylene manufacturing, and for the preparation of fatty acid esters with reduced crystallization temperature in biodiesel production (Lee et al., 1995).

In this study, P450PMOR2 was used as a model of biotechnologically useful P450 biocatalyst to identify metabolic engineering strategies to increase the efficiency of glucose utilization in supplying reducing equivalents to support a heterologous P450 reaction in *E. coli*. An experimental platform was set up for accurately measuring $Y_{PPG}$ in the absence of potential limiting factors or biases. Using this system, the performance of engineered *E. coli* strains, prepared with the purpose of increasing the intracellular pool of NADPH available to the P450 enzyme, was investigated. A P450PMOR2 derivative with dual NADPH/NADH cofactor specificity was also engineered in order to establish whether this property can increase the propanol-per-glucose yield by enabling the enzyme to recruit reducing equivalents from both the NADPH and NADH pools in the cell.

## Materials and Methods

### Strains, Plasmids, and Protein Purification

Strains and plasmids used in this study are listed in Table I. P450s were expressed from pCWori-based vectors containing the P450 gene under the control of a double tac promoter (BamHI/EcoRI cassette). Amino acid mutation in P450PMOR2 and 1–3 were reported previously (Fasan et al., 2007). The dual cofactor specificity dcl-12G was derived from 1-12G (Peters et al., 2003) by randomization of

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<th>Strain/plasmids</th>
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position R966, K972, Y974, and W1046 followed by screening for increased activity in the presence of NADPH and NADH on hexylmethyl ether (Peters et al., 2003). To prepare dcP450PMOR2, the C-terminal portion on hexylmethylether (Peters et al., 2003). To prepare dcP450PMOR2, the C-terminal portion

Engineered Strains

The engineered E. coli strains were prepared using W3110 as parent strain. Pgi and Zwf were prepared by P1 phage transduction using single gene deletion K-12 strains from the Keio collection (Baba et al., 2006). The other strains were prepared using the recombination method with phage λ-red recombination (Datsenko and Wanner, 2000). Plasmid pKD46 was used to express the recombination. To prepare NdhNuo, W3110(Δndh) and W3110(ΔnuoA-N) were first prepared by transforming W3110(pKD46) with the PCR product obtained using pKD13 template (Datsenko and Wanner, 2000) with primers ndhF and ndhR for W3110(Δndh) and primers nuoA_NF and nuoA_NR for W3110(ΔnuoA-N). After removing the FRT-flanked kan gene in W3110(Δndh) with FLP recombination (Causey et al., 2003), the ΔnuoA-N deletion was transferred into this strain using P1 phage transduction, resulting in NdhNuo. W3110(ΔldhA) was constructed by transforming W3110(pKD46) with the PCR product obtained using primers ldhA_ko_f and ldhA_ko_r and pKD13 as template. W3110(ΔadhE) was constructed by transforming W3110(pKD46) with the PCR product obtained using primers adhE_ko_f and adhE_ko_r and pKD13 as template. NdhNuoAdhE was prepared by first removing the kan gene from NdhNuo and then transducing ΔadhE mutation from W3110(ΔadhE). NdhNuoAdhE(KanR) was treated with FLP recombinase and then transduced with W3110(ΔldhA) P1 phage lysate, resulting in NdhNuoAdhLdh. Similarly, NdhAdhLdh was prepared starting from Ndh strain. NdhAdhLdhPgi and NdhAdhLdhPtaAchA were prepared transforming NdhAdhLdh(pKD46) cells with the PCR product obtained using primers pgi_KO_for/pgi_KO_rev and ackA-pta_KO_for/ackA-pta_KO_rev, respectively. All chromosomal deletions were verified by colony PCR and sequencing. Sequences of the primers are provided in the Supplementary Information.

Whole-Cell Reactions

Biotransformations were carried out in a 100 mL-fermentor at 25°C using a 1:1 mixture of propane and air at a flow rate of 5 L h⁻¹, and a glucose concentration of 2 mM maintained through feeding at 30-min intervals. Total propanol (1- and 2-propanol) produced was determined by GC according to described analytical procedures (Fasan et al., 2007).

Catalytic and Kinetic Properties of the P450 Variants

The catalytic properties of the P450 variants for NADPH- and NADH-driven propane oxidation were measured according to described methods (Fasan et al., 2007). Kinetic parameters for NAD(P)H oxidation were determined by steady-state kinetic analysis using the cytochrome P450 reduction assay (Neel et al., 2005). Reactions were carried using 20 nM P450 and 50 μM L⁻¹ bovine cytochrome c. Initial cytochrome P450 reduction rates were measured over 20 s using an ε₅₅₀ = 22,640 M⁻¹ cm⁻¹. Kₘ and kₗₐₜ values were estimated from least-squares nonlinear regression to the Michaelis–Menten equation from saturation plots of vₐ (initial rate) versus NAD(P)H concentration. All activity and kinetic measurements were performed at least in triplicate.
Results

Experimental System for Accurate Measurement of $Y_{PPG}$

Various factors may affect $Y_{PPG}$ analysis in whole-cell reactions involving oxygenases. In addition to potential substrate- or product-dependent toxicity and substrate availability limitations (Buhler et al., 2008; Walton and Stewart, 2004), the need for molecular oxygen for activity, cofactor uncoupling, and potential instability of these biocatalysts could be problematic (Li et al., 2002; van Beilen et al., 2003). Initial efforts were directed at developing an experimental platform for measuring $Y_{PPG}$ where potentially limiting factors are minimized.

Whole-cell reactions were carried out using suspensions of P450-expressing E. coli cells (wild-type W3110 and derivatives thereof) exposed to a 1:1 mixture of propane and air using nitrogen-free minimal medium. The nitrogen-free medium helped maintain the cells in a metabolically active, non-growing status where NAD(P)H-utilizing processes required for cellular growth are suppressed and a larger fraction of NAD(P)H becomes available to drive heterologous NAD(P)H-dependent reactions (Walton and Stewart, 2002). Under these conditions, the effects of gene deletions can be studied in the context of a simplified model of E. coli metabolism (Fig. 1). The use of non-growing cells also eliminated potential biases in $Y_{PPG}$ analysis due to differences in growth rate (and thus background energy demand) among the engineered E. coli strains.

Analysis of the in vivo activity of P450PMOR2 expressed in W3110 cells revealed a sharp decrease in the biocatalyst/strain performance at cell densities higher than 1.1 g cdw L$^{-1}$ (OD$_{600}$ > 5) (Fig. 2A), presumably as a result of a sub-optimal supply of oxygen to the biocatalyst and/or the cell. A cell density of ~0.4 g cdw L$^{-1}$ (OD$_{600}$ ~ 2) was thus utilized to minimize the effect of oxygen transfer on the $Y_{PPG}$. Substrate transfer rate was not expected to be limiting because of the eight-fold higher solubility of propane in water (~1.6 mM) compared to oxygen, the lack of endogenous processes competing for this substrate, and the low amount of propane consumed per unit of time under the applied conditions (up to 0.02 mM min$^{-1}$). Saturating levels of propane in the reaction medium also allowed for near-saturation of P450PMOR2 and the other P450BM3 variants investigated, whose $K_M$ for propane was previously determined to be between 150 and 300 µM (Fasan et al., 2008).

In previous studies, the intracellular concentration of folded P450PMOR2 in whole-cell biotransformations was found to decrease over time (Fasan et al., 2007), which was attributed to oxidative self-inactivation of the biocatalyst, a phenomenon observed in whole-cell reactions with other oxygenases as well (van Beilen et al., 2003; Walton and Stewart, 2004). Since the biocatalyst cannot be replenished under non-growing conditions, $Y_{PPG}$ were calculated over

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**Figure 1.** Schematic representation of E. coli central metabolism illustrating the major pathways implicated in the metabolism of NAD(P)H under aerobic, non-growing conditions. The heterologous P450-dependent reaction investigated in this work is highlighted (box). The genes targeted for deletion are noted in italics: ackA = acetate kinase; adhE = acetaldehyde dehydrogenase; pta = phosphotransacetylase; zwf = glucose-6-phosphate-1-dehydrogenase. EMP: Embden-Meyerhof-Parnas pathway, PP: pentose phosphate pathway, ED: Entner-Doudoroff pathway, TCA: tricarboxylic acid. Endogenous THD activity in E. coli is dependent on a membrane-bound (pntAB) and a soluble pyridine nucleotide THD (sthA) (dashed box). In the scheme, the reaction catalyzed by pyruvate oxidase (poxB) is also indicated.
providing NADPH for P450PMOR2 catalysis, a strain (Gottschalk, 1986). To analyze the role of the former in to be the major sources of NADPH during glucose oxidation isocitrate dehydrogenase in the TCA cycle are considered phosphate (PP) pathway and the NADPH-dependent NADPH available to the P450 enzyme. In major pathways implicated in NAD(P)H metabolism due to cofactor uncoupling (Blank et al., 2008). compared to dehydrogenases (Buhler et al., 2008) possibly that oxygenases may have higher ''cofactor requirements'' (Cirino et al., 2006; Walton and Stewart, 2004), previous stoichiometries (2.3–4.2) were reported for NAD(P)H-

**Figure 2.** Factors influencing enzyme activity and $Y_{PPG}$ in P450-dependent whole-cell propane biotransformation as measured using P450PMOR2 in wild-type *E. coli* (W3110) under non-growing conditions. A. Dependence of the enzyme in vivo activity on the cell density. Values refer to activities measured over 30 min at 25 °C in whole-cell reactions using air/propane (1:1) feed and minimal M9 medium pH 7.2 supplemented with 20 mM glucose. One unit (U) = 1 μmol product (propanol) per minute; cdw = cell dry weight. B. Dependence of $Y_{PPG}$ on the glucose concentration as determined by the molar ratio between propanol produced and glucose consumed after 2 h at a cell density of 0.4–0.45 g cdw L$^{-1}$ (corresponding to OD$_{590}$ ~ 2.0 in the reaction vessel).

2 h. Within this time, the concentration of catalytically active P450 remained >70% of the initial value (prior to the reaction) as indicated by CO-binding assay on the cell lysate.

Under our experimental conditions, a progressive decrease in $Y_{PPG}$ and accumulation of acetate up to 2 mM was observed as the glucose concentration was increased from 1 to 4 mM (Fig. 2B). This was consistent with less efficient consumption of the energy source at higher glucose concentrations due to “metabolic overflow” (Akesson et al., 2001). A glucose feeding system was thus implemented to maintain glucose around 0.5 (±0.2) mM throughout the biotransformation, thereby minimizing the effect of metabolic overflow on the $Y_{PPG}$.

**Higher Propanol-Per-Glucose Yields Through Modification of the Glycolytic Pathway**

Using the protocols described above, the $Y_{PPG}$ with P450PMOR2 in wild-type *E. coli* (W3110) was found to be 0.52 ± 0.5 (Fig. 3A). While much higher product-glucose stoichiometries (2.3–4.2) were reported for NAD(P)H-dependent dehydrogenases and reductases (Chin et al., 2009; Cirino et al., 2006; Walton and Stewart, 2004), previous studies with flavin-dependent monoxygenases indicate that oxygenases may have higher “cofactor requirements” compared to dehydrogenases (Buhler et al., 2008) possibly due to cofactor uncoupling (Blank et al., 2008).

To improve the efficiency of *E. coli* in supporting P450 catalysis, W3110 variants were constructed (Table I), where the major pathways implicated in NAD(P)H metabolism were altered in order to increase the amount of intracellular NADPH available to the P450 enzyme. In *E. coli*, the pentose phosphate (PP) pathway and the NADPH-dependent isocitrate dehydrogenase in the TCA cycle are considered to be the major sources of NADPH during glucose oxidation (Gottschalk, 1986). To analyze the role of the former in providing NADPH for P450PMOR2 catalysis, a strain featuring a non-functional PP pathway (Zwf) was prepared. This strain was found to provide a $Y_{PPG}$ of 0.49 ± 0.07, a value only slightly lower than that observed with W3110. This suggests that the relative carbon flux passing through this pathway in resting W3110 cells is small (≤10%), probably reflecting the metabolic pattern of *E. coli* when grown under nitrogen-limited conditions (Hua et al., 2003) rather than in rich media (where this fraction is 20% and 45% (Csonka and Fraenkel, 1977; Sauer et al., 2004)).

Increasing glucose oxidation through the PP pathway was thus expected to increase significantly the overall amount of NADPH available to the P450 reaction. Strain Pgi (W3110 Δpgi), which lacks the enzyme catalyzing the committed step of the glycolytic pathway (phosphoglucone isomerase), was prepared and found to support an $Y_{PPG}$ of 1.33 ± 0.21 (Fig. 3A), which indicated that the single gene deletion enabled a 155% more efficient utilization of glucose for the production of propanol as compared to wild-type *E. coli*.

**Removal of Intracellular NADH-Oxidizing Processes Increases $Y_{PPG}$**

Another potential source of NADPH in *E. coli* is through the endogenous transhydrogenase (THD) activity and, in particular, through the membrane-bound PntAB THD, which is believed to catalyze the reversible reduction of NADP$^+$ by NADH using the membrane proton motive force (Hua et al., 2003; Sauer et al., 2004). We reasoned that removing some of the endogenous NADH-utilizing processes would favor transfer of reducing equivalents from NADH to NADPH through the endogenous PntAB-dependent THD activity of the host. To this end, we targeted the major cellular processes contributing to re-oxidation of this cofactor, namely the reactions catalyzed by the NADH dehydrogenases (NDH-I and NDH-II) in the respiratory chain and the fermentation pathways responsible for the production of ethanol and lactate (Fig. 1). First, we evaluated the effect of eliminating both membrane-bound NADH dehydrogenases (strain NdhNuo). Deletion of these enzymes was expected to reduce *E. coli* respiration, thereby reducing competition with the P450 biocatalyst for both reducing power and for oxygen. The $Y_{PPG}$ obtained with strain NdhNuo was almost identical to that of the reference strain (0.55 vs. 0.52, respectively). Despite similar $Y_{PPG}$, a drastic increase in the amount of secreted fermentation products (primarily lactate and ethanol) was observed with NdhNuo (Fig. 3B), suggesting that the surplus of NADH equivalents is re-directed towards the fermentative pathways rather than towards the production of additional NADPH, perhaps because of the more favorable kinetic parameters of lactate and alcohol dehydrogenases (Shone and Fromm, 1981; Yin and Kirsch, 2007) compared to PntAB (Hu et al., 1995). Based on this result, another strain was tested where in addition to ΔnuoA-N and Δndh deletions, the genes responsible for lactate and ethanol production had also been removed (strain NdhNuoAdhLdh). This genetic modification resulted in
about a two-fold increase in $Y_{PPG}$ (0.98 ± 0.21) and, consistent with the genotype, only acetate accumulated in the medium as the major secreted byproduct during biotransformation.

While elimination of respiratory and fermentative reactions did result in improved propanol yield, significant quantities of acetate were also produced, which is indicative of overflow metabolism. Effects resulting from insufficient NAD$^+$ regeneration in glucose-driven metabolism include inhibition of pyruvate dehydrogenase and citrate synthase (Gottschalk, 1986). We therefore tested whether NAD$^+$ regeneration rates could be elevated to an intermediate level to help alleviate NADH-dependent regulation. Since NADH-II (encoded by ndh) is the predominant form of NADH dehydrogenase under aerobic conditions and its NADH oxidation activity tends to be highly uncoupled to proton translocation (Calhoun et al., 1993; Unden and Bongaerts, 1997), a strain lacking ndh in combination with Dldh and DadhE was prepared (strain NdhAdhLdh). This resulted in a considerable improvement in $Y_{PPG}$ (1.71 ± 0.17), indicating that the altered metabolism of NdhAdhLdh enables a 230% more efficient utilization of glucose to drive the heterologous P450-dependent reaction than possible with wild-type E. coli.

In an attempt to further increase the $Y_{PPG}$ by recovering the glucose-derived energy dissipated through acetate secretion by NdhAdhLdh (Fig. 3B), the genes encoding for phosphotransacetylase ($pta$) and acetate kinase ($ackA$) were also removed, resulting in strain NdhAdhLdhPtaAckA. The $Y_{PPG}$ obtained with this strain however was only 0.71 ± 0.11, and large quantities of pyruvate were secreted as a result of the new deletions (Fig. 3B), which had not been detected with any of the other strains. The accumulation of pyruvate in cultures of $pta$-ackA-deficient strains has been observed before (Yang et al., 1999).

As the best performing strain among the NADH dehydrogenase-deficient variants, NdhAdhLdh was then used as parent strain to introduce the Δpgi mutation.
previously found to be beneficial. However the YPPG obtained with the resulting strain (NdhAdhLdhPgi) did not improve compared to NdhAdhLdh (1.58 ± 0.08 compared to 1.71 ± 0.17). Despite similar YPPG, the metabolite profiles and the glucose uptake rates of the two strains showed notable differences. The amount of acetate accumulated in the reaction with NdhAdhLdhPgi was <20% of that observed with the NdhAdhLdh strain. Also, detectable quantities of the TCA cycle intermediate succinate were observed with NdhAdhLdhPgi. Compared to NdhAdhLdh, NdhAdhLdhPgi showed a considerably reduced (−60%) glucose uptake rate (0.34 vs. 0.89 mmol g⁻¹ cdw h⁻¹). A similar effect (−67%) was observed after deletion of the pgi gene in the wild-type strain (0.32 mmol g⁻¹ cdw h⁻¹ for Pgi compared to 0.99 mmol g⁻¹ cdw h⁻¹ for W3110, Fig. 3C). The negative effect of Δpgi on the E. coli glucose uptake rate has been observed previously (Canonaco et al., 2001; Chin et al., 2009; Sauer et al., 2004), and it is enhanced when cells are grown under nitrogen-limited conditions (Hua et al., 2003).

While the in vivo activity of P450pMO R2 in W3110 cells at low glucose concentration (0.5 mM) is lower than that measured at high glucose concentration (20 mM) (9.2 ± 1.3 (1 h) vs. 43.6 ± 5.0 U g⁻¹ cdw (0.5 h)), the measured YPPG under the former conditions is about 2.5-fold higher (0.5 vs. 0.2) indicating that glucose is utilized more efficiently. Across the strains (Fig. 3D), the increase in YPPG resulting from the gene deletions was reflected by an increase in the biocatalyst in vivo activity with the exception of the strains carrying the Δpgi deletion, whose glucose uptake rates are reduced compared to the others.

**P450 Propane Monoxygenase With Dual NADH/ NADPH Cofactor Specificity**

Next, we reasoned that a P450 enzyme which can utilize both NADPH and NADH would harvest reducing equivalents more readily than a strictly NADPH-dependent isomorph, possibly eliminating potential energy losses and overcoming thermodynamic limitations associated with THD activity (Sauer et al., 2004). To test this hypothesis, we prepared a P450pMO R2-derived variant with dual cofactor specificity. First, studies were conducted to confirm that P450pMO R2 is exclusively driven by NADPH as P450B3 is. Despite two mutations near the NADPH-binding site (D698G and I710T), P450pMO R2 was found to share with P450B3 similar K₅ values for NADPH and NADH (Table II). Also, P450pMO R2 produced no propanol in the presence of 4 mM NADH, which far exceeds the physiological levels of this cofactor in E. coli (Bennett et al., 2009). To engineer a P450pMO R2 variant with dual cofactor specificity, the amino acid residues primarily involved in NADPH recognition were targeted for mutagenesis (Fig. 4).Switching of P450BM3 cofactor specificity has been reported (Maurer et al., 2005; Neeli et al., 2005). Our goal however was to obtain a P450 variant capable of utilizing both NADPH and NADH at physiological relevant conditions. A set of amino acid substitutions (R966N, K972H, Y974F, and W1046D) were introduced into P450BM3 (J.A. Rodriguez and F.H. Arnold, unpublished results). The introduction of these mutations into P450pMO R2 led to a variant that catalyzes either NADPH- or NADH-driven propane oxidation with good catalytic rates (122 and 144 turnovers/min, respectively) and coupling efficiency (76% and 62%, respectively) (Table II). Importantly, the K₅ values of P450pMO R2 for NADPH and NADH were determined to be between 3- and 10-fold lower than the expected concentration of these cofactors in E. coli (Bennett et al., 2009), indicating that this enzyme can support both NADPH- and NADH-dependent propane oxidation in vivo (Table II).

YPPG values were measured with P450pMO R2 in W3110 and the other strains, resulting in values from 30% to 50% lower than those obtained with P450pMO R2. This difference is likely due to the lower coupling efficiency and less favorable catalytic rate of P450pMO R2 compared to P450pMO R2. To better evaluate the impact of cofactor

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**Table II.** Catalytic and kinetic properties of the P450B3 variants.

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<td>K₅ (μM)</td>
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<tr>
<td>k₅ (min⁻¹)</td>
<td>2,460 ± 175</td>
<td>1,440 ± 25</td>
<td>6,050 ± 115</td>
<td>6,010 ± 180</td>
</tr>
<tr>
<td><strong>NADH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propanol rate (min⁻¹)</td>
<td>0</td>
<td>144 ± 12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Couplingb</td>
<td>0</td>
<td>62%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K₅ (μM)</td>
<td>8,350 ± 380</td>
<td>52.5 ± 3.0</td>
<td>14,900 ± 650</td>
<td>6,230 ± 260</td>
</tr>
<tr>
<td>k₅ (min⁻¹)</td>
<td>4,390 ± 340</td>
<td>1,650 ± 200</td>
<td>3,870 ± 70</td>
<td>2,130 ± 120</td>
</tr>
</tbody>
</table>

aInitial (20 s) propanol formation rates as determined by GC.
bCoupling efficiency as given by the ratio between propanol formation rate and cofactor (either NADPH or NADH) consumption rate in propioni

Previously found to be beneficial. However, the YPPG obtained with the resulting strain (NdhAdhLdhPgi) did not improve compared to NdhAdhLdh (1.58 ± 0.08 compared to 1.71 ± 0.17). Despite similar YPPG, the metabolite profiles and the glucose uptake rates of the two strains showed notable differences. The amount of acetate accumulated in the reaction with NdhAdhLdhPgi was <20% of that observed with the NdhAdhLdh strain. Also, detectable quantities of the TCA cycle intermediate succinate were observed with NdhAdhLdhPgi. Compared to NdhAdhLdh, NdhAdhLdhPgi showed a considerably reduced (−60%) glucose uptake rate (0.34 vs. 0.89 mmol g⁻¹ cdw h⁻¹). A similar effect (−67%) was observed after deletion of the pgi gene in the wild-type strain (0.32 mmol g⁻¹ cdw h⁻¹ for Pgi compared to 0.99 mmol g⁻¹ cdw h⁻¹ for W3110, Fig. 3C). The negative effect of Δpgi on the E. coli glucose uptake rate has been observed previously (Canonaco et al., 2001; Chin et al., 2009; Sauer et al., 2004), and it is enhanced when cells are grown under nitrogen-limited conditions (Hua et al., 2003).

While the in vivo activity of P450pMO R2 R2 in W3110 cells at low glucose concentration (0.5 mM) is lower than that measured at high glucose concentration (20 mM) (9.2 ± 1.3 (1 h) vs. 43.6 ± 5.0 U g⁻¹ cdw (0.5 h)), the measured YPPG under the former conditions is about 2.5-fold higher (0.5 vs. 0.2) indicating that glucose is utilized more efficiently. Across the strains (Fig. 3D), the increase in YPPG resulting from the gene deletions was reflected by an increase in the biocatalyst in vivo activity with the exception of the strains carrying the Δpgi deletion, whose glucose uptake rates are reduced compared to the others.
specificity on \( Y_{PPG} \) without biases due to differences in coupling properties, the performance of dcP450\_PMO\_R2 in W3110, Zwf and Pgi strains was compared with that of variant 1–3 (Fasan et al., 2007), which shares with dcP450\_PMO\_R2 similar coupling efficiency in propane oxidation and \( K_M \) for NADPH (Table II). The \( Y_{PPG} \) values obtained with dcP450\_PMO\_R2-expressing cells were found to be higher than those obtained with 1–3 (Fig. 5A). These results indicate that the NADPH/NADPH-utilizing variant is indeed able to retrieve extra reducing equivalents compared to the strictly NADPH-dependent isozyme. Interestingly, the increase in \( Y_{PPG} \) varied with the genotype of the host, being relatively large in Zwf (+90%), moderate in W3110 (+40%), and small in Pgi (+20%). This likely reflects a difference in the relative levels of NADPH and NADH between these strains. The much lower performance of 1–3 compared to P450\_PMO\_R2 also highlighted the impact of the coupling efficiency on the \( Y_{PPG} \), considering that these P450 variants have comparable propane turnover rates and \( K_M \) for NADPH (Table II).

As evinced from the data in Figures 3C and 5B, the glucose uptake rate of the strains did not appear to be significantly affected (±20%) by the type of P450 variant expressed in the cell. Importantly, the metabolite profiles observed in the whole-cell reactions with dcP450\_PMO\_R2 and P450\_PMO\_R2 were virtually identical. With NdhNuo, acetate, lactate, and ethanol concentrations were 0.56 (±0.20), 0.45 (±0.18), and 0.25 (±0.14) mM for dcP450\_PMO\_R2 and 0.58 (±0.14), 0.48 (± 0.21), and 0.23 (± 0.09) mM for P450\_PMO\_R2, respectively. The negligible differences in the amount of secreted metabolites even in the presence of excess NADH (as in NdhNuo) suggested that dcP450\_PMO\_R2 competes only poorly with the endogenous lactate dehydrogenase and aldehyde/alcohol dehydrogenase for NADH oxidation.

**Discussion**

P450-dependent biotransformations have attracted much interest as environmentally friendly strategies for carrying out oxidations of synthetic relevance (Chang et al., 2007; Pandey et al., 2010; Sowden et al., 2005; van Beilen et al., 2005). Analyzing the propanol-per-glucose yield in P450\_PMO\_R2-dependent whole-cell reactions, we determined that the native metabolic network of _E. coli_ is fairly inefficient in supporting the activity of the heterologous P450 enzyme through glucose oxidation (\( Y_{PPG} \approx 0.5 \)). Since P450\_PMO\_R2 features “native-like” catalytic properties (high coupling, turnover rate and total turnover numbers (Fasan et al., 2007) and a \( k_{cat}/K_M \) for propane oxidation of \(~5 \times 10^4 \text{M}^{-1} \text{s}^{-1}(\text{Fasan et al., 2008})\)) this yield is probably representative of those achievable in biotransformations involving natural P450 monooxygenases and possibly other NAD(P)H-dependent oxidases (Buhler et al., 2008; Walton and Stewart, 2004).

In this study, the three strategies investigated to improve this yield were: (a) directly increasing NADPH production in the cell by increasing flux through NADPH-producing reactions (PP pathway); (b) indirectly increasing NADPH production (via endogenous THD activity) by eliminating competition for NADH (\( \Delta ndh, \Delta nuoA-N, \Delta ldh, \) and \( \Delta adhE )\); and (c) using a dual-cofactor-specificity mutant of the P450 propane monooxygenase. While all these approaches led to appreciable increases in the \( Y_{PPG} \) the use of a NADPH-dependent propanoxygenase and an engineered _E. coli_ strain with the triple knockout \( \Delta ndh, \Delta adhE, \) and \( \Delta ldh (\text{strategy “b”}) \) provided the best solution among those tested, enabling a 3.3-fold more efficient utilization of glucose to drive the P450 reaction compared to W3110.

Increasing the carbon flux through the PP pathway is often pursued (Chin et al., 2009; Lim et al., 2002; Poulsen et al., 2005; Skorupa Parachin et al., 2009) or proposed (Walton and Stewart, 2004) as a strategy to increase the efficiency of NADPH-dependent reactions in whole-cell systems. Our results indicate that while the \( \Delta pgi \) mutation improves the \( Y_{PPG} \) (2.6-fold), this benefit is counterbalanced by a decrease in the specific in vivo activity of the biocatalyst (Fig. 3D), which in the case of P450\_PMO\_R2 drops from 8.0 ± 0.3 U g\(^{-1}\) cdw in W3110 to 4.5 ± 0.1 U g\(^{-1}\) cdw in Pgi as a result of the much reduced glucose uptake rate of the latter strain (Fig. 3C). In contrast, the altered metabolism of strain NdhLdhAdhE resulted in a 3.3-fold higher \( Y_{PPG} \) without altering significantly the glucose uptake rate of the host, which thus translated into a proportionally large increase in the in vivo activity of the biocatalyst (24.6 ± 2.2 U g\(^{-1}\) cdw). The effect of \( \Delta pgi \) mutation on the

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**Figure 4.** Engineering of P450\_PMO\_R2 NADPH-binding site to produce a propane monooxygenase with dual cofactor specificity. **A.** Three-dimensional representation of P450\_BM3 structure prepared using the crystal structure of the heme and FMN domain (Li and Poulos, 1997) and a homology model of P450\_BM3, FAD-binding domain (Fasan et al., 2007). Arrows indicate the prosthetic groups and cofactors of the enzyme: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate. **B.** Enlarged view of the region of P450\_BM3 targeted for mutagenesis (corresponding to the boxed area in panel **A**). Dual cofactor specificity was achieved by mutating K972, Y974, and W1046, which are primarily involved in NADPH binding as indicated by sequence alignment (Neeli et al., 2005) and inspection of P450\_BM3, reductase domain model (Fasan et al., 2007). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]
glucose uptake rate of the strain and on the catalyst in vivo activity is also apparent from the studies with 1–3 and csP450PMOR2 (Fig. 5).

Removing respiratory NADH dehydrogenases proved effective in increasing the \( Y_{PPG} \) of the propane biotransformations. In particular, eliminating only the \( ndh \) gene (strain NdhAdhLdh) resulted in a yield that was 70% higher than that of the strain having deletions in both \( ndh \) and \( nuo \) (NdhNuoAdhLdh). It is interesting to note that despite the difference in \( Y_{PPG} \), the level of acetate produced by these two strains is similar, which suggests that more reducing power is made available for propanol production in NdhAdhLdh, even though glucose oxidation was not increased. One possible explanation is that in NdhNuoAdhLdh a greater portion of acetate production proceeds through pyruvate oxidase (PoxB) (Fig. 1), in which reducing equivalents are delivered directly to the electron transport chain, rather than via pyruvate dehydrogenase which generates an additional NADH. Another explanation may be that the relative PP pathway and glycolytic fluxes differ between the two strains, resulting in different NADPH versus NADH production ratios.

The higher yields of strains NdhNuoAdhLdh and NdhAdhLdh compared to NdhNuo also indicate that eliminating fermentative routes is important for improving NADPH supply, either by reducing competition with the THD for NADH or by directly re-routing carbon flux through NADPH-producing reactions (e.g., in the PP pathway). The latter possibility is supported by the observation that removal of \( pgi \) in NdhAdhLdh does not further improve the \( Y_{PPG} \). Activation of the glyoxylate shunt, and thus reduced NADPH output from the TCA cycle, has been observed in phosphoglucose isomerase-deficient \( E. coli \) and this may contribute to the lower yields observed with NdhAdhLdhPgi (Hua et al., 2003). It is also possible that as a result of the \( \Delta pgi \) mutation the THD reaction becomes

![Figure 5](image_url). Side-by-side comparison of the performance of W3110, Zwf, and Pgi strains expressing either the NADPH-utilizing P450 propane monoxygenase 1–3 or the NADPH/NADH-utilizing variant dcP450PMOR2. A: Propanol-per-glucose yields as measured after 2 h. B: Glucose uptake rates of the strains expressed in mmol glucose per gram cdw per hour. C: In vivo propane oxidation activity of the P450 variants as measured after 1 and 2 h. 1 U = 1 μmol propanol min⁻¹.
thermodynamically limited from further increasing NADPH levels in the cell. While calculation of the exact number of reducing equivalents channeled to the P450 reaction in these strains is not possible due to uncertainty with respect to the coupling efficiency of the monoxygenase in vivo, we can conclude that the additional amount of NADPH made available for the P450 reaction as a result of the triple gene deletion in NdhAdhLdh corresponds to more than two times that available in the wild-type strain.

With dcP450PMOR2, we determined that a P450 with dual cofactor specificity can lead to relative $Y_{\text{PPG}}$ improvements of up to 90% compared to a strictly NADPH-dependent isoform. The benefit provided by the relaxed cofactor specificity, however, appears to be off-set by the less favorable catalytic properties of this variant compared to P450PMOR2, making E. coli cells expressing dcP450PMOR2 a comparatively less efficient biocatalytic system. The relatively poor affinity of dcP450PMOR2 for NADH also likely limits its NADH-dependent activity in vivo. A NADH/NADPH-utilizing P450 variant with more favorable kinetic and coupling properties is therefore expected to provide even higher yields than currently achieved.

Although a glucose feeding strategy was implemented to minimize overflow metabolism, $Y_{\text{PPG}}$ values in this study could be significantly improved by reducing secretion of glucose byproducts and more completely oxidizing glucose. For example, quantification of secreted metabolites (ethanol and acetate) for strains W3110, Pgi, and NdhAdhLdh reveals that about 50%, 10%, and 25% of glucose carbon, respectively, is lost through fermentation rather than being further oxidized through the TCA cycle. With fermentative pathways deleted in strain NdhAdhLdhPtaAckA, large amounts of pyruvate were instead secreted. Even when correcting for the unobtainable reducing equivalents present in secreted byproducts, however, the resulting $Y_{\text{PPG}}$ calculations appear to be lower than theoretically achievable. Similar results were reported for the case of NADPH-dependent xylose reduction to xylitol during glucose oxidation in the absence of acetate secretion (Cirino et al., 2006) or when correcting for secreted reducing power (Chin et al., 2009). Reducing metabolic competition for reducing equivalents, overcoming NADH-dependent metabolic regulation, and eliminating metabolic inefficiencies leading to “wasted” energy are all strategies expected to further improve reduced cofactor regeneration for whole-cell biocatalysis.

In summary, we report gene knock-out combinations which can significantly increase the product-per-glucose productivity of E. coli for P450PMOR2-dependent reactions. Insights were gained with respect to features of the host and biocatalyst that could be modified to achieve further improvements in the $Y_{\text{PPG}}$. These analyses will help guide metabolic and protein engineering strategies for improving the performance of this and other biotechnologically important P450-dependent biotransformations in E. coli.

This work was supported by USDA grant number 2006-35505-16660 (F.H.A.) and by the NSF grant number BES-0519516 (P.C.C.). This material is also based upon work supported by the U.S. Department of Defence Contract No. DAAD19-02-D-0004.

References


Supplemental information

Sequences of the primers used for the chromosomal gene deletions:

nhF
5'-ATACACCCCTCACTCTATATCACTCTACAATATCCGCTCAGTGAGGCTGGAGCTGCTTC-3'

ndhR
5'-ATGCAACTTTCAACCCGGACGATAACCGCCTTAATACCTGCTATCCCGGGATCGGCTGACC-3'

nuoA_NF
5'-TTCATCGCATCGGACGATGATACATTTGAGACAATAGTGTGAGGCTGGAGCTGCTTC-3'

nuoA_NR
5'-CATCAGCCGCTTGCAAACGCACAATGCTAATCAGCGGATGTTCCCGGGATCGGCTGACC-3'

ldhA_ko_f
5'-TGTGATTCAACATCAGCTCGGAGATAATCTGCTATCCCGGGATCGGCTGACC-3'

ldhA_ko_r
5'-TTGCAAGCGTGACGGAATATCAGGTAGCTTTCTGCTATCCCGGGATCGGCTGACC-3'

adhE_ko_f
5'-GTTATCTAGTTGTGCAAACATGCTAATGCGGAACAAATCGTGGAGGCTGGAGCTGCTTC-3'

adhE_ko_r
5'-GCAGTTTCACCTTTCTACATATCAGCAGGCAGATTCCGGGGATCGGCTGACC-3'

pgi_KO_for
5'-CGGCTAAGGGTTTACACTCAAACATTACGCTAATCCGACTAACCATAATCAAACCCTACTAAAGGGCG-3'

pgi_KO_rev
5'-CACGCTTTATAGCGGTAAATCAGGACATTAGCTGCGCTGTGGAATACGACTCTAGTGGGCTC-3'

ackA-pta_KO_for
5'-CCATACCACGACTTCATGGATCTCCCTGAGCTGGAAGGCTAAATAGCTACAAATCCCTGCTAAAAGGGCG-3'

ackA-pta_KO_rev
5'-CTGCTGCTGCTGCTGAGACTGGAATCAGCGAGCAGATGAGGTAGCTGGAATACGACTGACTATAAGGGCTC-3'